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Establishment of efficient protocol *in-vitro* Shoot Regeneration from Nodal Explants. *Withania somnifera* (L.) Dunal

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Abstract

The Present investigation has given a method for *in vitro* micropropagation of a valued medicinal plant *Withania somnifera*. In this study we used nodal part of *Withania somnifera as* explants. Explants were cultured on MS media (Murashige and Skoog) supplemented with different concentrations of Growth hormones like BAP (0.1-2.0mg/l) NAA (0.1-1.0mg/l). The excellent shoot induction followed by 1mg/l BAP+ 0.5mg/l NAA in this combination was observed as 90% and maximum shoot length was recorded (4.08cm.). Regenerated shoots were rooted on MS medium supplemented with IBA (0.1-1.0) mg/l and NAA (0.1-1.0) mg/l. maximum root length (6.cm) contributed by IBA at 1.0 mg/l on MS medium.

Key words: *In vitro*, micropropagation, *Withania somnifera*, medicinal plant.

Abbreviation: BAP : 6-Benzyllaminopurine
NAA: Naphthalene acetic acid
KN : kinetin
MS : Murashige and Skoog medium),
Mg : Mile gram
PGR : Plant growth regulators composition

Contents

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1. Introduction

Medicinal plants are of great interest to the researchers in the field of biotechnology as most of the drug industries depend in part on plants for the production of pharmaceutical compounds **(Chand *et al 1997.),*** *Withania somnifera* commonly knows us ashwagangadha belong to Solanaceae family. *W. somnifera* is normally propagated by seeds.

The wall of the fruit contains a chemical which prevent seed germination. The most important part used in medicine is the tuber or root. It is important Ayurvedic drug of India. The plant had been reported to grow in wild and is also cultivated in selective areas of India. Their pharmacological properties are diverse ranging from anti-inflammatory,anti-tumor, anti-stress, anti-oxidant, immunomodulatory, hemopoetic and cardio-protective effects. Its root part rich in alkaloids (withanine) (Majumdar, 1955). *Withania help on* female disorders, cough, rheumatism and dropsy (Kiritikar and Basu, 1975) roots, the other parts of this plant also useful for the treatment of inflammatory conditions, tuberculosis and exhibits excellent antitumor and anti-bacterial activities (Devi and Sharada, 1992, Devi, 1996). The leaves contain withanolides like withaferin A that exhibit anti-bacterial and anti-tumor properties (Devi and Sharada, 1992; Devi, 1996). *W. somnifera* an important medicinal shrub is being exploited on a large scale on a commercial basis for its medicinal value. Because of over exploitation, this plant is becoming a member in the endangered plant species (Antonisamy and Manickam, 1999). Micropropagation is an useful tool for conservation and multiplication of several medicinal plant**s** (Sivanesan, 2007b; Sivanesan and Jeong, 2007a, b).

This species propagates easily by seeds but does not have the natural ability for regenerative propagation, and hence the variability generated by sexual recombination is difficult to fix (Jayanti and Sharma, 1991). And its *in vitro* micro propagation is therefore very necessary. So the plant requires conservation to meet its demand in agriculture and medicine. The bio-technological approach such as plant tissue culture is an alternate and variable method for propagation and conservation of economically and medicinally important plants. The media composition and qualitative and quantitative aspects of plant growth regulators play a vital role in micro propagation. Therefore, optimization of these conditions is a prerequisite for *in-vitro* related work. There are very few reports available on *in-vitro* propagation of *Withania somnifera* that made us interested to develop micro propagation protocol for this valuable medicinally plant species *In vitro* propagation of this useful medicinal plant could provide a means of dis-ease free healthy clones for extraction of pure drugs. The present study was undertaken to affect of different types of plant growth hormones a protocol for in-vitro micro propagation of *Withania somnifera to* regenerate plants by using different explants by tissue culture for Micro propagation to meet its demand in medicine and agriculture.

2. Materials and Methods

The research was conducted at the tissue culture laboratory, Research and Development for Greenery environment (RDGE) 148 plot industrial areas Govindpura Bhopal, Madhya Pradesh, India.

Collection and authentication of plant material

The planting material (shoots) were collected in the month of May, 2013 from mature plant growing inside the garden of tissue culture laboratory, Research and Development for Greenery environment (RDGE) 148 plot industrial areas Govindpura Bhopal,

Selection of explants

Apical shoot buds & meristems were used as explants for this experiment. Explants were cut and reduced to length of 2 cm using surgical blade, retaining the apical dome (1 cm).

Surface sterilization procedure

Shoots were thoroughly washed under running tap water for 30 min to remove all the dirt and soil particles adhering to them. then treated with 5% tween-20 for 5 minutes with constant stirring followed by 3-4 rinses in sterile distilled water and further treated with an antifungal agent (Bavistin) for 1 hours and were further with detergent for 15 min. and rinsed 4-5 times tap water. Thereafter, again explants were kept immersed in distilled water with few drops of wetting agent, labolene for ten minutes. It was immediately followed by five time rinses in distilled water to remove traces of labolene.

Further sterilization procedures were carried out inside laminar air flow chamber, where shoots were surface sterilization through 1 min. treatment in 70% (v/v) for half minute followed by three times rinses in sterile distilled water. There after mercuric chloride (0.1%) treatment was given to explants for 2 minutes followed by four times rinsed in sterile distilled water. Thereafter shoots were carefully transferred to be placed over sterile Petri plats to remove excess water & were then inoculated into the culture establishment medium (MS) using sterile forceps under aseptic conditions.

Chemicals and glass wear

Apical shoot bud & meristems induced from shoots were cultured on MS basal medium supplemented with 3 % (w/v) sucrose (Sd-fine Chemicals, India) for shoot induction. The pH of the medium (supplemented with respective growth regulators) was adjusted to 5.7 with 1N NaOH or 1N HCl before gelling with 0.8 % (w/v) agar. In all the experiments, the chemicals used were of analytical grade (Merck and SD-fine Chemicals, India).The medium was dispensed into culture vessels (Borosil, Mumbai, India) and autoclaved at 105 kPa at 125°C for 15 minute. The surface sterilized explants were placed vertically on the culture medium. All the cultures were incubated at 25±2°C under 16h light/8h dark photoperiod with irradiance of 45 - 50 µ mol/ m²/s photo synthetically active radiation

(PAR) provided by cool white fluorescent tubes (Philip, India) and with 60 - 65 % relative humidity. All subsequent subcultures were done at four weeks intervals. Culture media consisted of MS(Murashige(1962) supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar (Himedia, India) was evaluated for their effects on *in-vitro* growth and development of. For *in-vitro culture* of shoots, explants were cultured on MS medium supplemented with different concentration of cytokines, including BAP (0.1-2.0mg/l) NAA (0.1-1.0mg/l) either individually or in combination. Application of tissue culture to plant conservation in India has been largely restricted to economically important species However, the approach could usefully be extended to conserve all threatened plants so that vital biodiversity and the ecological network is sustains can be preserved **(Jiten C.2011)**

Shoot induction

Apical shoot tip and meristems node were excised and inoculated by vertical orientation on the culture medium containing different concentration of BAP (0.1-2.0mg/l) NAA (0.1-1.0mg/l). Ten single explants were assigned randomly to each treatment and culture were kept under 16 h light/day photoperiod at 25±2°C.shoot induction the effects of different treatments were quantified and the data were subjected to presented. Medium lacking growth regulators served as control.

Shoot multiplication

The explants with bud proliferation cultures were transferred to fresh MS media BAP (0.1-2.0mg/l) NAA (0.1-1.0mg/l) with various supplements' for shoot multiplication; the cultures were maintained by regular subculture on fresh medium with the same composition.

Rooting

Multiple plants separately and transferred into rooting media IBA (0.1-1.0) mg/l and NAA (0.1-1.0) mg/l. for root induction.

3. Results and Discussion

Results and Discussion

Different concentrations of growth hormones tested like auxin (BAP, IBA, and NAA) and cytokinin (BAP), the Combination and their concentration were mentioned in table. The present study exemplifies a positive modification of shoot induction efficacy on MS with combination of auxin and cytokinins (BAP, NAA) were producing maximum number of shoots and longer shoot length. Excision and culture of the nodal segments from *in vitro* derived shoots facilitated the development of increased number of shoots.

The excellent shoot induction followed by 1mg/l BAP+ 0.5mg/l NAA in this combination was observed as 90% and maximum shoot length was recorded (4.08 cm.)., after two weeks of incubation. Regenerated shoots were rooted on MS medium supplemented with IBA (0.1-1.0) mg/l and NAA (0.1-1.0) mg/l. maximum root length (6.99cm) contributed by IBA at 1.0 mg/l on MS medium Multiple shoots start arising from the nodal explants after 2 weeks of incubation. Micropropagation has many advantages over conventional methods of vegetative propagation, which suffer from several limitations (Nehra *et al*., 1994).

The plant growth regulators not only control the shoot bud formation but also influence the root induction.The effect of NAA and IBA on root induction was carried out. Root formations have been recorded within 7 days in MS media supplemented with 1.0 mg/L NAA and 1.0 mg/l IBA. Among them maximum number of roots (7.00) and root length (5.21cm.) of the plant. In the present study we developed a simple and efficient protocol for direct regeneration from nodal explants of *Withania somnifera* . *In-vitro* propagation of plants holds tremendous potential for the production of high-quality plant-based medicines (Murch *et al*., 2000). The protocol can be exploited for commercial propagation and conservation of potential endangered medicinal plant resources.

Table 1. Effects of different concentration of plant growth regulators on *in-vitro* shoot initiation from apical shoot of *Withania somnifera.* After 4 weeks of culture

S.No	PGR.Contration(Mg/l)	% of shoot induction
1.	0.0b	0%
2	0.1BAP	%
3	0.5BAP+0.1NAA	30%
4	0.5BAP+0.5NAA	50%
5	1.0BAP	70%
6	1mg/l BAP+ 0.5mg/l NAA	90%
7	1.5mg/l BAP+ 0.5mg/l NAA	60%
8	2mg/l BAP+ 2.0mg/l NAA	50%
9	2mg/l BAP+ 2.0mg/l NAA	40%

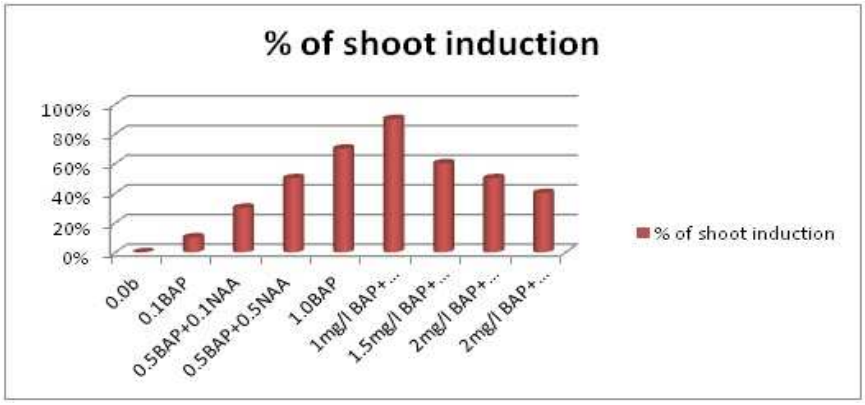

Figure.1

Table 2. Effects of different concentration of plant growth regulators on *in-vitro* shoot multiplication of *Withania somnifera.* After 4 weeks of culture

PGR Code	PGR.Contration(Mg/l)	% of shoot multiplication	Shoot length cm	Number of shoot
(A)	2.0 BAP+1.0KN	55	1.63	3-5
(B)	2.0BAP	55	1.63	3-5
(C)	1.5BAP+2.0NAA	65	2.65	4-5
(D)	1.5BAP+0.5NAA+2.0KN	70	2.95	4-5
(E)	2.0NAA+1.0KN	30	3.63	5-6
(F)	2mg/l BAP+ 0.5mg/l NAA+1.0KN	85	4.08	8-10
(G)	3mg/l BAP+ 1.5mg/l NAA1.5KN	55	2.08	7-8
(H)	4mg/l BAP+ 2.0mg/l NAA	40	1.05	2-3
(I)	5mg/l BAP+ 1.5mg/l NAA+2.0KN	40	05.08	2-3
(J)	5mg/l BAP+ 2.0mg/l NAA+2.0KN	30	04.08	2-3

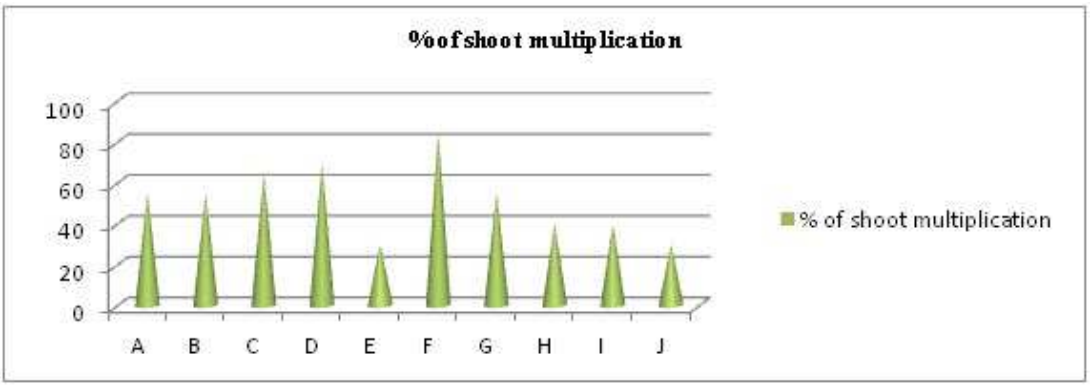

Figure.2 Multiplication

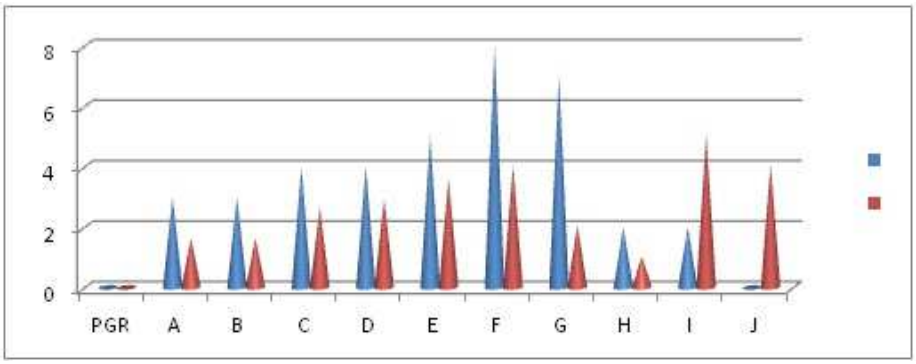

Figure.3 shoot length &no

Table 3. Different growth regulators for Rooting response of *Withania sominefera*

PGR.Contration(Mg/l)	No. of roots	Root length (cm)
0.0b	1-2	1-2
0.1NAA	3-4	1-2
0.1IBA+0.1NAA	5-6	2-3
0.5IBA+0.5NAA	6-5	5-6
1.0 IBA	7-8	5-6
1.0IBA+ 0.5NAA	3-4	4-5
1.0 NAA	2-3	4-5
1.0 NAA+0.5IBA	3-4	3-4
1.0 BAP+ 1.0 NAA	3-4	3-4

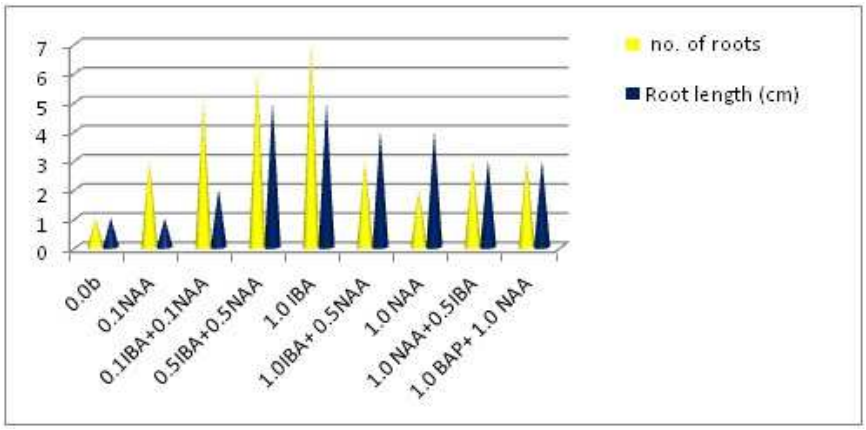

Figure.4

Figure.6 Natural plant

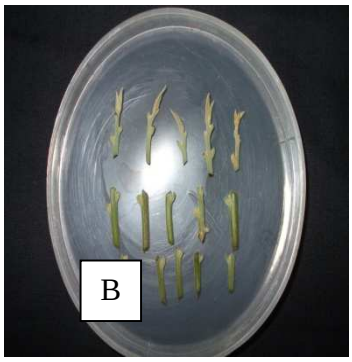

Figure.7 Explants

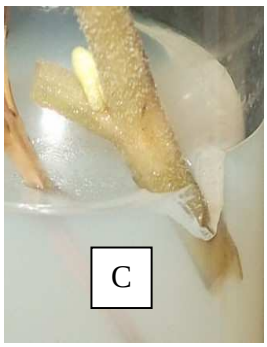

Figure.8 Bud induction

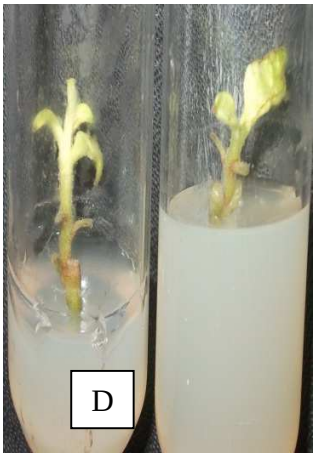

Figure.9 Shoot induction

Figure.10 Multiplication

Figure.11 Best Root induction

4. Conclusion

Different plant growth regulators clearly effected shoot induction from axillaries and meristum through of *in-vitro* micropropogation. It can be concludes that the 1mg/l BAP+ 0.5mg/l NAA are showed high% of shoot induced compare by other combination. The protocol defined in this study as outlined below and is demonstrated in figgers. The findings have several implications for managing the diversity of this species as well as restoration of its degradation. The developed protocol can be used to produce uniform and desirable plants for plantation in order to reduce pressure on the wild population. It also affairs a potential system that should be used for improvement conservation and mass propagation of *Withania somnifera***.** conservation and production of this plant can be a challenge as well as a powerful tool to medicinal properties improve livelihoods and enhance biodiversity. The present experiment have show that it is possible to *in vitro* micropropogation and use for further experiments' and done by plantlets**.**

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